CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Elsevier - Publisher Connector

FEBS 28398 FEBS Letters 566 (2004) 201–206

Neocarzinostatin naphthoate synthase: an unique iterative type I PKS from neocarzinostatin producer *Streptomyces carzinostaticus*

Basundhara Sthapit[a], Tae-Jin Oh[a], Rajan Lamichhane[a], Kwangkyoung Liou[a], Hei Chan Lee[a], Chun-Gyu Kim[b], Jae Kyung Sohng[a,*]

[a] *Institute of Biomolecule Reconstruction (iBR), Department of Chemistry, Sun Moon University, #100, Kalsan-ri, Tangjeong-myeon, Asansi, Chung-Nam 336-708, Republic of Korea*
[b] *Department of Pharmaceutical Engineering, Inje University, 607 Obang-dong, Kimhae City, Kyungnam 621-749, Republic of Korea*

Received 13 March 2004; revised 7 April 2004; accepted 7 April 2004

Available online 28 April 2004

Edited by Stuart Ferguson

Abstract Enediyne antibiotics are known for their potent antitumor activities. One such enediyne, neocarzinostatin (NCS), consists of a 1:1 complex of non-peptide chromophore (1a), and peptide apoprotein. The structurally diverse non-peptide chromophore is responsible for its biological activity. One of its structural components, the naphthoic acid moiety (2,7-dihydroxy-5-methyl-1-naphthoic acid, 1d) is synthesized by a polyketide synthase (PKS) pathway through condensing six intact acetate units. The 5.45 kb iterative type I PKS, neocarzinostatin naphthoate synthase (NNS), responsible for naphthoic acid moiety biosynthesis, shares sequence homology with 6-methyl salicylic acid synthase of fungi and orsellinic acid synthases (AviM and CalO5) of *Streptomyces* origin. Cultures of *S. lividans* TK24 and *S. coelicolor* YU105 containing plasmids with NNS were able to produce 2-hydroxy-5-methyl-1-naphthoic acid (2a), a key intermediate of naphthoic acid moiety in NCS. In addition to 2a, a novel product, 2-hydroxy-5-hydroxymethyl-1-naphthoic acid (2d) was isolated. This is the first report of a bacterial iterative type I PKS from an enediyne producer which enables the biosynthesis of bicyclic aromatic compounds.
© 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

Keywords: 2-Hydroxy-5-methyl-1-naphthoic acid; Heterologous expression; Iterative type I PKS; Neocarzinostatin; Neocarzinostatin naphthoate synthase

1. Introduction

Nature has provided a variety of structurally diverse polyketides which have been used as valuable therapeutic agents. Much interest has been taken in searching for and developing novel polyketides of clinical significance. Polyketides are biosynthetically derived from simple metabolites like acetates, malonates, propionates, butyrates and their derivatives. Polyketide synthase (PKS) catalyzes a decarboxylative condensation of these compounds into the growing polyketide chain. Microbial PKSs are classified into three major types. A type I PKS system consists of one or more multifunctional proteins that contain a different active site for each enzyme-catalyzed reaction in polyketide carbon chain assembly and modification [1]. The macrolides of erythromycin and avermectin are examples of type I PKS products. Type II PKSs, on the other hand, comprise sets of iteratively used individual proteins for the production of multicyclic aromatic compounds (e.g., actinorhodin and tetrocenomycin) [2]. Type III PKSs are small homodimer proteins that belong to the chalcone and stilbene synthase superfamily [3]. Beside these typical PKS, there exists a multifunctional monomodule PKS which catalyzes iterative condensation of extenders in a polyketide chain. They belong to a separate class, named as iterative type I PKS. The representative examples are those involved in the biosynthesis of 6-methyl salicylic acid (6-MSA) in fungi (6-MSAS) [4] and orsellinic acid synthase in bacteria (AviM) [5].

Enediynes, the most potent antitumor antibiotics known so far, are also synthesized by a PKS pathway. They are characterized by either a nine-membered bicyclo [7.3.0] dodecadiynene or a ten-membered bicyclo [7.3.1] tridecadiynene "warhead" as a highly reactive core appended with aryl and sugar moieties. The recent gene cluster cloning and identification of warhead PKS in the biosynthesis of C-1027 [6] and calicheamicin [7] opened a new avenue for enediyne biosynthesis research. Furthermore, analysis of putative gene clusters from several enediyne producing actinomycetes, isolated by a rapid PCR based genome scanning approach, shows a conserved enediyne gene cassette including warhead PKS. It suggests that biosynthesis of all enediyne warhead may proceed via similar polyketide intermediates [8,9].

The chromophore of highly potent nine-membered neocarzinostatin (NCS) consists of four different moieties, namely the warhead (epoxybicyclo [7.3.0] dodecadienediyne), naphthoic acid, *N*-methyl fucosamine and an ethylene carbonate unit (Fig. 1(a)). The chromophore is protected by non-covalent binding within the apoprotein. The mechanism of chromophore binding to apoprotein has been elucidated [10] and the expression, characterization and functional identification of apoprotein have also been carried out [11]. Additionally, the detailed mechanism of single- and double-stranded DNA cleavage by NCS in vitro has been extensively investigated.

* Corresponding author. Fax: +82-41-544-2919.
E-mail address: sohng@sunmoon.ac.kr (J.K. Sohng).

Abbreviations: 6-MSA(S), 6-methyl salicylic acid (synthase); ACP, acyl carrier protein; AT, acyl transferase; DH, dehydratase; HPLC, high performance liquid chromatography; KR, ketoreductase; KS, ketosynthase; NCS, neocarzinostatin; NNS, neocarzinostatin naphthoate synthase; ORF, open reading frame; PKS, polyketide synthase; TLC, thin layer chromatography

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.04.033

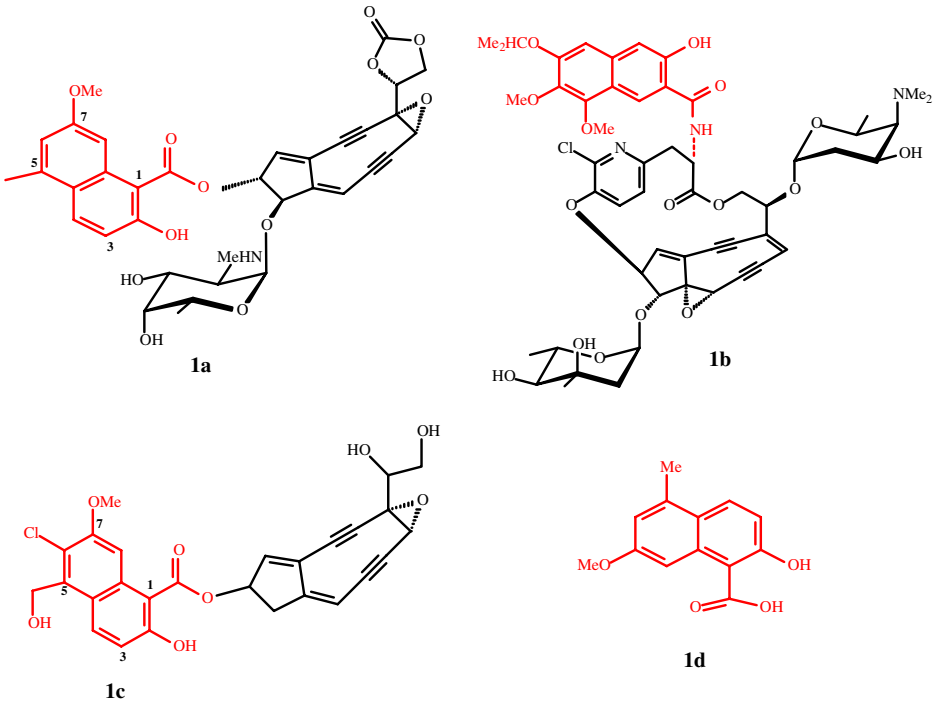

Fig. 1. Structurally diverse chromophore of: (a) NCS (b) kedarcidin (c) N1999A2 and (d) naphthoic acid moiety of NCS. Naphthoic moieties are highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A thiol induced warhead binds to DNA in the minor groove and rearranges to form a bicyclic diradical that abstracts a hydrogen atom from deoxyribose sugar rings [12,13]. Myers et al. proved the role of sugar moiety as trivial in the specificity towards DNA bases and proposed that the planar naphthoate moiety (Fig. 1(d)) may have a crucial role [14]. On the other hand, Goldberg and co-workers have proposed a model that involves the intercalation of naphthoate moiety between bases of DNA with simultaneous positioning of the warhead within the minor groove, which then abstracts C-5′ hydrogen via a free-radical mechanism [15].

A ^{13}C labeled feeding experiment proved that naphthoate moiety is formed from six intact acetate units linked in head to tail fashion [16]. Evidence from homology we report here of neocarzinostatin naphthoate synthase (NNS) to 6-MSAS, which is most extensively studied as iterative type I PKS [4,29] involved in the biosynthesis of 6-MSA, leads us to predict acetyl-CoA as the sole initiation unit and five malonyl-CoA units as extenders that condense to give the products. Furthermore, NNS shows homology to AviM involved in the biosynthesis of orsellinic acid moiety in avilamycin [5] and to CalO5 (an iterative type I PKS putatively involved in the synthesis of aryl moiety in calicheamicin) [7]. Since 6-MSAS, AviM and CalO5 are involved in the synthesis of monocyclic aromatic compounds, the NNS involved in the biosynthesis of bicyclic aromatic compounds is significant. It is of interest to understand and investigate the molecular basis of this very unusual iterative type I PKS.

We cloned and sequenced an approximately about 33.0 kb genomic DNA fragment of *Streptomyces carzinostaticus* in a cosmid clone selected from screening. Analysis of the DNA sequence revealed the presence of NNS along with some sugar and accessory genes. In the course of the present work, Ben Shen and co-workers (Division of Pharmaceutical Science and Department of Chemistry, University of Wisconsin-Madison, US) also reported the gene cluster of NCS which was deposited in GenBank under the accession number AY117439. Our sequence data are in full agreement. However, our analysis of the position of the most probable ribosome binding site has caused us to place the start codon of NNS 189 bp upstream from that currently assigned in Genebank.

We describe here the cloning and functional identification of NNS by in vivo heterologous expression in *S. lividans* TK24 and *S. coelicolor* YU105. The product isolation and characterization resulted in two compounds, namely 2-hydroxy-5-methyl-1-naphthoic acid (**2a**) and 2-hydroxy-5-hydroxymethyl-1-naphthoic acid (**2d**). Compound **2a** is a key intermediate of naphthoic acid moiety of NCS, while **2d** is a shunt product probably produced by the action of some host enzyme(s) upon primary metabolite **2a**. This report represents the first isolation and characterization of naphthoate synthase from enediyne producing *Streptomyces*.

Substituted naphthoate moieties are also found in some other nine-membered enediynes like kedarcidin (Fig. 1(b)) produced from *Streptoalloteichus* sp. [17] and N1999A2 (Fig. 1(c)) produced from *Streptomyces* sp. AJ9493 [18], indicating the possibility of finding NNS homology genes in them.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Streptomyces carzinostaticus ATCC 15944 was maintained on ISP2 agar plates. Genomic DNA was prepared after growing cells in YMB medium at 28 °C for three days. *Escherichia coli* XL1 Blue MRF was used for gene manipulation and preparation of recombinant plasmids. *E. coli* ET 12567/pUZ8002 was used as a host to prepare unmethylated

plasmid DNA. Liquid LB medium was used for *E. coli* cultures. *S. lividans* TK24 and *S. coelicolor* YU105 were maintained on R2YE agar plates at 28 °C and for protoplast preparation the cells were grown into YEME media with 5 mM $MgCl_2$ and 0.5% Glycine at 28 °C for 36 h. For product isolation, *S. lividans* TK24 and *S. coelicolor* YU105 harboring the expression plasmids were cultivated in R2YE liquid media. Ampicillin (100 μg/ml), thiostrepton (12.5, 50 μg/ml), and apramycin (100 μg/ml) were used for the selection.

2.2. Construction of genomic library and screening

Cloning and transformation of competent *E. coli* cells and in vitro DNA manipulations were carried out according to the standard protocols [19]. Chromosomal DNA was isolated from *S. carzinostaticus* after lysozyme treatment and phenol–chloroform extraction as described by Hopwood and co-workers [20]. Then, it was partially digested with *Sau*3A and fragments of 30 to 40-kb were ligated into the cosmid vector pOJ446 (which had been digested with *Hpa*I and *Bam*HI sites). The ligated products were packaged with Gigapack III XL system (Stratagene, USA) and transduced into *E. coli* XL1 Blue MRF. Primers were designed from the conserved region of dTDP-glucose 4,6-dehydratase genes from several actinomycetes and NCS apoprotein gene. The 340 bp DNA fragment corresponding to dTDP-D-glucose 4,6-dehydratase gene from the genome of *S. carzinostaticus* was amplified using primers DW11 (upstream): 5′-CACTTCGGGGGC-GAGTCGCA CGT-3′and DW32 (downstream): 5′-GGGCCGT-AGTTGTTCGAGCA-3′. Similarly, 196 bp DNA fragment of NCS apoprotein gene was amplified using the primers APO-1 (upstream): 5′-TGGGAATTCG CGGTGGTGCAGT-3′and APO-2 (downstream): 5′-GCAGATCTAGACCGCCTACGACG-3′. These PCR products were ligated into pGEM-3Zf(−) vector, sequenced and taken as probes for library screening. In hybridization experiments, the probes were labeled with digoxigenin (DIG) using a DIG labeling and detection kit (Boehringer, Mannheim, Germany) and purified by gel filtration. Hybridization was carried out for 6 h with each probe at 50 °C in 10 ml of 2 × SSC [19]. Colony and Southern hybridization were performed with Hybond N nylon membranes (Amersham, Braunschweig, Germany) according to standard protocols. One cosmid clone, pNEO32, that hybridized with both probes was further confirmed by Southern hybridizing with *eryA*II module-3 gene from *Saccharopolyspora erythraea* and taken for sequencing.

2.3. DNA sequencing and computer analysis

Restriction enzyme digested DNA fragments of approximately 200–3000-bp of pNEO32 were subcloned into pGEM3, pGEM7, and pBluescript SK(−) vectors. Sequencing was performed by the dideoxynucleotide chain termination method on automatic sequencer. The DNASIS software package (version 2.1, 1995; Hitachi Software Engineering, San Bruno, CA) and BLAST (NCBI) were used for sequence analysis and homology search in the GenBank database, respectively.

2.4. Construction of expression vector and plasmid

A high copy number expression vector pSE34, a derivative of pWHM3 with bidirectional *ermE** promoters [21], was modified by destroying the *Eco*RI site and introducing a multiple cloning site (MCS) towards one of the promotors. A new synthetic MCS was designed using a pair of complimentary oligonucleotides 5′-GAT-CCCTGCAGTCTAGACCATGGGAATTCCTCGAAG-5′, which were annealed and cloned into *Bam*HI/*Hin*dIII digested pSE34 vector to create pIBR25. Whole NNS gene was cloned into pIBR25 in two steps. The first 558 bp were amplified from pNEO32 using primers BS1 (forward): 5′-GTGCTGCAGACGGGCCGAATCTGTC-3′ and BS2 (reverse): 5′-GTCGTTGGAATTCGCGGCGG-3′ (restriction sites underlined). The PCR product was cloned into *Pst*I/*Eco*RI digested pIBR25 to form pNBS1. Then, 6.0 kb *Eco*RI fragment of the gene from pNEO32 was inserted into the same site of pNBS1 to create pNBS2.

2.5. Protoplast preparation, transformation and screening

Polyethylene glycol mediated pNBS2 transformation into *S. lividans* TK24 and *S. coelicolor* YU105 was carried out as described by Hopwood and co-workers [20]. The transformed host cells were regenerated on R2YE agar plates, overlaid with soft agar containing 12.5 μg/ml of thiostrepton and screened on R2YE plates with 50 μg/ml thiostrepton. Parallel transformation and screening were also carried out with pIBR25.

2.6. Extraction and isolation of the products formed by NNS

The hosts transformed with pNBS2 and pIBR25 were inoculated into a 250 ml baffled flask containing 50 ml R2YE liquid media with 50 μg/ml thiostrepton and grown at 28 °C for 7 days. Then, whole content was extracted with 100 ml ethyl acetate after adjusting pH to 3.5 using 1 M HCl. The organic phase was separated and evaporated under vacuum. For preparative scale, 2000 ml culture media were used under the same conditions.

2.7. Thin layer chromatography (TLC), ESI- MASS, high performance liquid chromatography (HPLC) and NMR analysis

TLC of crude extract was carried out on aluminum silica plates (25DC Alufolien, Kieselgel 90F_{254}, MERCK, Germany) with MeOH/$CHCl_3$/C_6H_{14}/HCOOH (8:80:5:1) as a solvent. Developed plates were charred with sulfuric acid reagent. The compounds were purified by Silica column (Kieselgel 60, MERCK, Germany) chromatography with a gradient elution of C_6H_{14} and $CHCl_3$ with 0–30% MeOH solvent system. Further analysis was carried out by HPLC with a UV detector at 260 nm. Mightysil RP-18, GP 250-4.5 (5 μm) column was used in a linear gradient of 0–40% CH_3CN in H_2O at a flow rate of 1.0 ml/min. The pure fractions collected from silica column were further analyzed by ESI-MASS (Finnigan TSQ 7000 Mass Spectrometer). Purified compounds were subjected to NMR spectroscopy (Varian, FT-NMR 300 MHz, Unity Inova) after dissolving in DMSO-d_6.

3. Results

3.1. Cloning of biosynthetic gene cluster

On the assumption that the genes for the chromophore lie adjacent to the gene for the apoprotein, we adopted a strategy of using the NCS apoprotein as a probe to find the biosynthetic gene cluster of NCS. The dTDP-D-glucose 4,6-dehydratase gene and type I PKS modular gene (*eryA*II) were also used as probes. We have sequenced about 33.0 kb of a cosmid clone (pNEO32) screened by three gene probes. A total of 33 open reading frames (ORFs) along with NNS were located in pNEO32. The analysis revealed putative regulatory, transport, structural, and unknown genes.

3.2. Sequence analysis of NNS

NNS shows homology with 6-MSAS, (38% identity), AviM (43% identity) and CalO5 (45% identity). Interestingly, they show almost identical domain to domain similarity to each other. The gene has putative ATG as start and TAG as stop codons, flanked by genes for *O*-methyltransferase (*O*-MT) and ATP-dependent adenylate/thioester-forming enzyme (A) (Fig. 2). Comparative analysis of the acyl transferase (AT) domain homology of NNS with other modular and iterative type I PKS revealed an acetate specific signature. It contains the

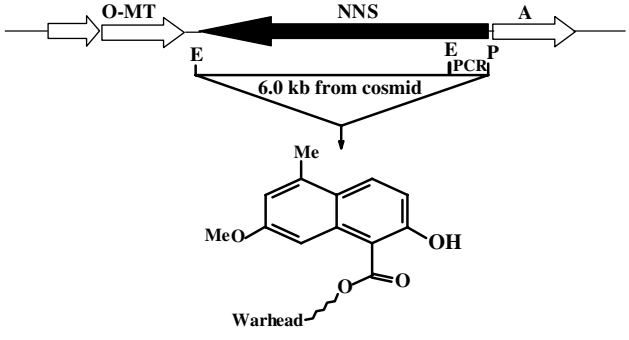

Fig. 2. Strategy for cloning NNS into the expression vector pIRB25. E, *Eco*RI; P, *Pst*I; *O*-MT; A, ATP dependent adenylate/thioester-forming enzyme.

active site Ser[676] residue in a conserved motif [674]GHSVG[678], typical for binding selectivity of CoA esters of dicarboxylic acid [22]. At the C-terminal, 100 amino acids downstream from the active Ser, four other proposed invariant residues with conserved Ala and His in the consensus motif [776]**A**F**HS**[779] are identified. A Phe adjacent to a His residue is found to be crucial for malonyl-CoA AT [23]. (It is Ser for methyl malonyl-CoA AT and Gln for ethyl malonyl-CoA AT [24].) The ketosynthase (KS) domain belongs to the thiolase family (Pfam: PF00108) having N-terminal active site residue Cys[228] in the consensus motif of [225]DTA**C**AGAL[232]. Also, the His[363/403] residues at C-terminal are found as in other modular PKSs; histidines act as general bases to increase the nucleophilicity of an active Cys residue [23]. The C-terminal of NNS has a shorter acyl carrier protein (ACP) with Ser[1759] active site in a motif of [1755]LGVD**S**VMTV[1763] that binds phosphopantetheine, where extenders attach. Unlike AviM and CalO5, it has a ketoreductase (KR) domain (1434–1614 amino acids) with a N-terminal NADP(H) binding motif [1448]**GL**G**C**L**G**LSVA[1457] showing homology of 53% identity with that of 6-MSAS from *P. patulum*. Since other modular PKSs have His, Gly and Pro active site residues in conserved domain of the type **H**XXX**G**XXXX**P** [25], a region with the motif of [982]**H**GLV**G**VEVT**P**[991] between AT and KR domains in NNS is assigned as a dehydratase (DH) domain.

3.3. Cloning of NNS into an expression vector

It was not possible to clone the NNS gene into an expression vector in one step. Therefore, the first few bps of the gene were amplified with suitable restriction sites, and remaining portion was taken directly from the cosmid (Fig. 2). The correct constructs were identified by restriction enzyme analysis.

3.4. TLC, ESI- MASS, HPLC and NMR analysis

After transformations, colonies which were morphologically different from wild type of hosts were observed. The sporulation was completely diminished and a huge amount of brown pigment was produced in agar plates. TLC of the crude extract showed two distinct UV fluorescent spots which were not observed in the crude extract of control transformants containing pIBR25. Silica gel column chromatography afforded 12.0 mg/l of purified **2a** and 7.0 mg/l of **2d**. The HPLC profile of crude extract of transformants show the retention time of 50.3 min and 26.6 min, respectively (Fig. 3), for the compounds **2a** and **2d** under the conditions mentioned in Section 2.7. The assignment on HPLC profile was further examined by ESI-MASS spectrometry. The major molecular ion peaks at 201.2 (m/z) for **2a** and 217.0 (m/z) for **2d** were observed. Finally, they were characterized by ^{1}H NMR and ^{13}C NMR spectroscopy.

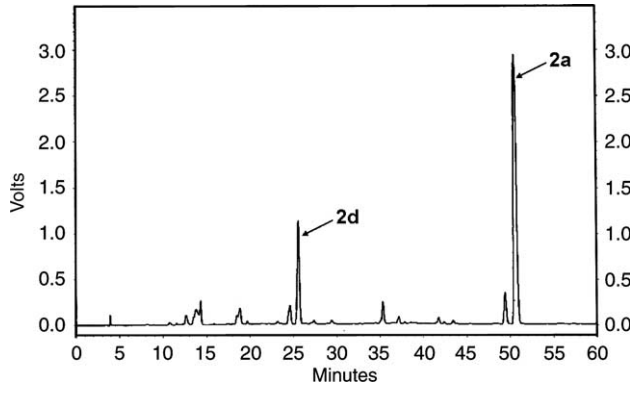

Fig. 3. HPLC profile of crude extract of pNBS2 transformants. **2a**, 2-Hydroxy-5-methyl-1-naphthoic acid; **2d**, 2-hydroxy-5-hydroxymethyl-1-naphthoic acid.

2a NMR data. ^{13}C NMR [$(CD_3)_2SO$, 75 MHz] δ 172.48 (C-1 CO_2H), 159.23 (C-2), 135.02 (C-5), 132.81 (C-4), 129.88 (C-9), 127.85 (C-7), 127.15 (C-10), 124.62 (C-6), 123.56 (C-3), 119.34 (C-1), 111.58 (C-8), 20.11 (C-5 Me). ^{1}H NMR [$(CD_3)_2SO$, 300 MHz] δ 2.66 (3H, s, Me-5), 7.16 (1H, d, $J = 7.00$ Hz, H-6), 7.17 (1H, d, $J = 9.20$ Hz, H-3), 7.37 (1H, q, 7.00/8.50 Hz, H-7), 8.02 (1H, d, 9.20 Hz, H-4), 8.37 (1H, d, $J = 8.60$, H-8), 10.57 (1H, br s, OH-C2).

2d NMR data. ^{13}C NMR [$(CD_3)_2SO$, 75 MHz] δ 172.18 (C-1, CO_2H), 158.07 (C-2), 139.03 (C-5), 132.35 (C-4), 129.92 (C-9), 127.89 (C-10), 125.95 (C-7), 124.38 (C-6), 122.73 (C-3), 119.08 (C-1), 111.73 (C-8), 62.13 (C5, CH_2OH). ^{1}H NMR [$(CD_3)_2SO$, 300 MHz] δ 4.90 (2H, s, –CH_2 C5), 7.20 (1H, d, $J = 9.20$, H-3), 7.37 (1H, d, $J = 7.00$, H-6), 7.40 (1H, q, $J = 7.00/8.60$, H-7), 8.12 (1H, d, $J = 9.20$, H-4), 8.22 (1H, d, $J = 8.37$, H-8).

4. Discussion

Biological activities of NCS are conserved in the chromophore region. The naphthoate moiety has a crucial role as described. Therefore, we sought to identify and characterize the NNS responsible for the biosynthesis of the naphthoate moiety. The sequence analysis shows homology with iterative type I PKSs of fungal and bacterial origin. Considering the consensus active motif of KS in modular and iterative type I PKSs, KS of NNS shows differences in some amino acids adjacent to active Cys residue (Fig. 4). Such differences may have some significance. Taking as an example of the evidence from the X-ray crystal structure of type II KS (ZhuH) of R1128 gene cluster [26], KS is responsible for controlling not only the choice of substrates, but also for the extension of polyketide chain. The side chains in several hydrophobic residues of KS, that make a pocket for accommodating an acyl-CoA chain, are determinants for controlling the carbon chain. This may equally be true for NNS. However, the actual assignment of chain length control by KS domain will have to await experimental verification. The conserved extender binding motif of ACP domain in NNS deviates from LGINS,

PikKS1 (PikAI)	1277 - VDTA	CSSSL	- 1285
TylKS1 (TylG)	1228 - VDTA	CSSSL	- 1236
NysKS1 (NysB)	205 - VDTA	CSSSL	- 213
OleKS1 (OleAI)	1219 - VDTA	CSSSL	- 1227
AviM	192 - IDTA	CSASL	- 200
CalO5	178 - VDTA	CSASL	- 186
6-MSAS	199 - VDAA	CASSL	- 208
NNS	224 - VDTA	CAGAL	- 232

Fig. 4. Alignment of the conserved active site amino acids of KS domains of various modular and iterative type I PKSs. KS1 represents extender KS. Pik, pikromycin (AF079138); Tyl, tylosin (SFU78289); Nys, nystatin (AF263912); Ole, oleandomycin (AAF82408); AviM, PKS in avilamycin (AAK83194); CalO5, iterative type I PKS in calicheamicin (AAM70355); 6-MSAS (P22367). Amino acids enclosed into a box show the difference. Numbers represent position of amino acids.

which is a typical case for most prokaryotes. Such deviation, i.e., LGXDS, is also found in some modular systems of avermectin [27] and candicidin biosynthesis [28].

As to the functional identification of NNS, the expected gene product 2,7-dihydroxy-5-methyl-1-naphthoic acid (**2b**) was not isolated. Instead, **2a** was recovered as a major product. A biosynthetic pathway catalyzed by NNS is thus proposed based on the production of the major compound synthesized (Fig. 5). By reference to the well established biosynthetic pathway of 6-MSAS from *P. patulum* [29,33], the ketoreduction and dehydration steps probably take place only after the condensation of 2nd and 4th malonyl-CoA, yielding the single hydroxylated product. It indicates that the hydroxylation at the C-7 position does not belong to a PKS path and instead may be carried out by certain hydroxylases. Available cytochrome P450 in the vicinity of NNS gene cluster is a probable candidate for this function. Later, the OH group undergoes *O*-methylation by the adjacent *O*-methyltransferase gene (*O*-MT) giving the final product **2c**. The other flanking gene for the ATP-dependent adenylate/thioester-forming enzyme will then activate **2c** to get it attached to warhead chromophore (Fig. 2). The final cyclization leading to the product formation is presumably a non-enzymatic reaction. The release of the product does not require a thioesterase, a feature characteristic of iterative type I PKS. It was reported that 6-MSA is released through ketene pathway [30].

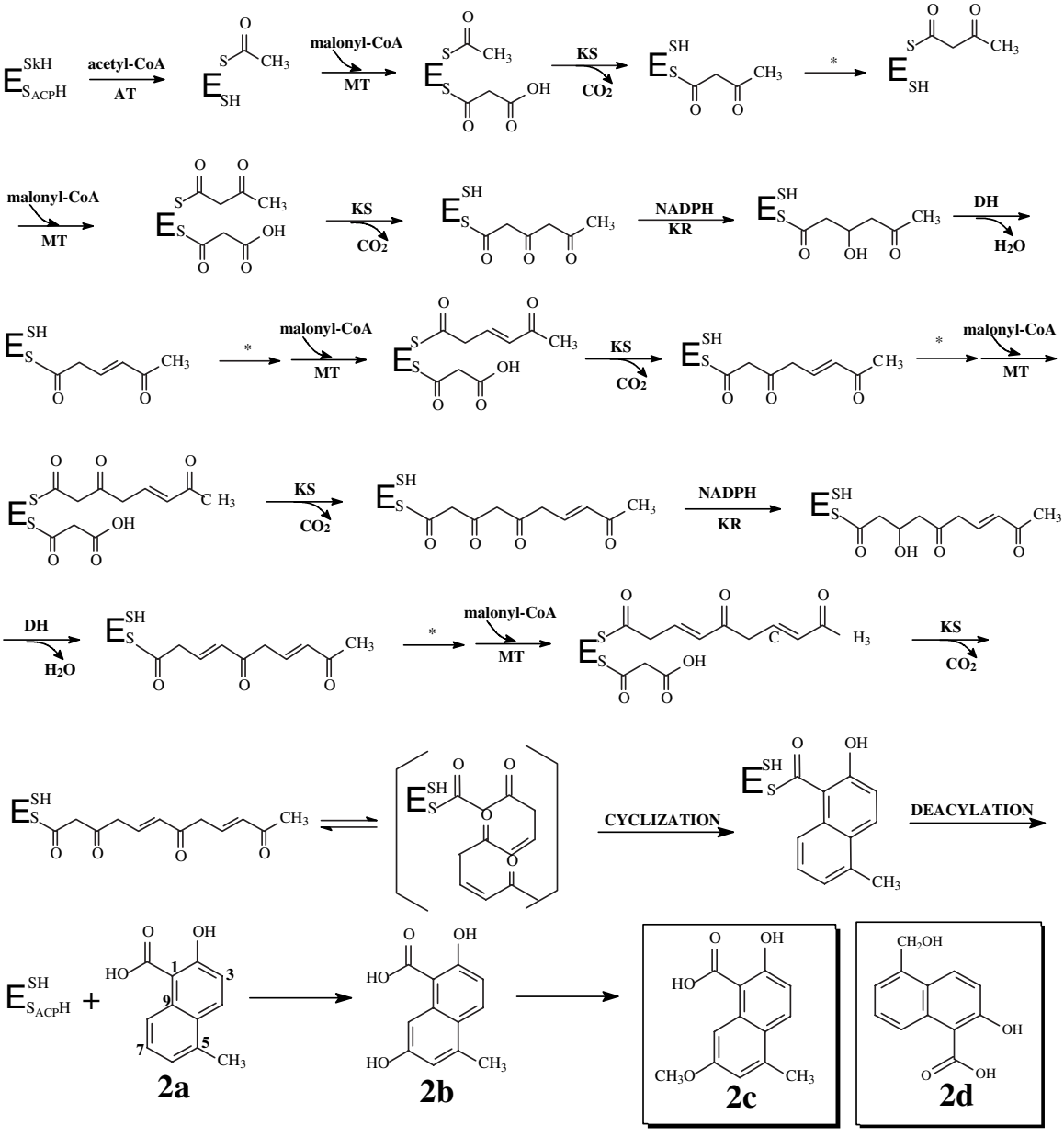

Fig. 5. Proposed biosynthetic pathway catalyzed by NNS from *S. carzinostaticus*. The loading of acetyl and malonyl units onto PKS (E) via KS (S_k) and prosthetic group of the ACP (S_{ACP}), respectively. KR, ketoreduction; DH, dehydration; AT, acetyl transferase; MT, malonyl transferase, *, transacylation from ACP to KS. Scheme based on Beck et al. [33].

The product synthesized by heterologously expressed PKS is sometimes accompanied by extra product(s) modified in their structure. They may be either intermediates or flexible priming products or products formed by the action of enzyme(s) in host cell. The examples are the production of HU235 by the expression of PKS genes related to R1128 biosynthesis [31] and pentaketide monocarboxylic acid production in the biosyntheis of 1,3,6,8-tetrahydroxynaphthalene by tetrahydroxynaphthalene synthase (T4HN) [32]. Production of the second compound **2d** is most likely by the action of a hydroxylase in host cell upon the primary metabolite **2a**. Though a probable mechanism of **2d** production cannot be given without experimental evidence, it is appropriate to mention that the naphthoic acid moiety of N1999A2 (which is a nine membered enediyne similar to NCS) has a $-CH_2OH$ group at the same position C-5 [16] (Fig. 1(c)). Thus, NNS should share a common mechanism to that of naphthoate moiety biosynthesis in N1999A2 for **2d** production.

Clinical use of NCS is restricted by instability and substantial toxicity. To obtain a more stable form of NCS with improved chemotherapeutic properties while retaining its biological activity is a challenge in NCS research. Alteration of the structure of the naphthoic acid moiety may be a tool in controlling such effects, since it is predicted to be involved in specificity of NCS action. Study of NNS at a molecular level elucidates the iterative chain formation mechanism in this novel enzyme. Engineering of it using a hybridization strategy like domain swapping with the domains of other PKSs accepting different kind of priming and extending units will definitely lead to the production of modified NCS.

Recent studies on bacterial aromatic polyketide biosynthesis are focused on altering product structure in several aspects like primer unit incorporation, chain length and so on. Thus, using the concept of combinatorial biosynthesis, a variety of novel polyketides can be biosynthesized along with NNS. Furthermore, this work demonstrates a considerable promise in identifying naphthoate moieties in the other case of enediyne antibiotics mentioned above.

Acknowledgements: This work was supported by the 21C Frontier Microbial Genomics and Application Center Program from the Korean Ministry of Science and Technology, Grant MG020302 to K. Liou. We thank Dr. Tokutaro Yamaguchi (Measurement Center, Division of Natural Science, Sun Moon University) for performing ESI-MASS and NMR.

References

[1] Staunton, J. and Wilkinson, B. (1997) Chem. Rev. 97, 2465–2497.
[2] Rawlings, B.J. (1999) Nat. Prod. Rep . 16, 425–484.
[3] Funa, N., Ohnishi, Y., Fujii, I., Shibuya, M., Ebizuka, Y. and Horinouchi, S. (1999) Nature 400, 897–899.
[4] Light, R.J. (1967) J. Biol. Chem. 242, 1880–1886.
[5] Gaisser, S., Trefzer, A., Stockert, S., Kirschning, A. and Bechthold, A. (1997) J. Bacteriol. 179, 6271–6278.
[6] Liu, W., Christenson, S.D., Standage, S. and Shen, B. (2002) Science 297, 1170–1173.
[7] Ahlert, J., Shepard, E., Lomovskaya, N., Zazopoulos, E., Staffa, A., Bachmann, B.O., Huang, K., Fonstein, L., Czisny, A., Whitwam, R.E., Farnet, C.M. and Thorson, J.S. (2002) Science 297, 1173–1176.
[8] Zazapoulos, E., Haung, K., Staffa, A., Liu, W., Bachmaan, B.O., Nonaka, K., Ahlert, J., Thorson, J.S., Shen, B. and Farnet, C.M. (2003) Nat. Biotechnol. 21, 187–190.
[9] Liu, W., Ahlert, J., Gao, Q., Pienkowski, E.W., Shen, B. and Thorson, J.S. (2003) Proc. Natl. Acad. Sci. USA 100, 11959–11963.
[10] Kim, K.H., Kwon, B.M., Myers, A.G. and Rees, D.C. (1993) Science 262, 1042–1046.
[11] Nozaki, S., Tomoiko, Y., Hishinuma, T., Inoue, M., Nagumo, Y., Tsuruta, L.R., Hayashi, K., Matsumoto, T., Kato, Y., Ishiwata, S., Itoh, K., Suzuki, T. and Mizu-gaki, M. (2002) J. Biochem. 131, 729–739.
[12] Dedon, P.C. and Goldberg, I.H. (1992) Biochemistry 31, 1909–1917.
[13] Kuo, H.M., Lee-Chao, P.D. and Chin, D.H. (2002) Biochemistry 41, 897–905.
[14] Myers, A.G., Kort, M.E. and Hammond, M. (1997) J. Am. Chem. Soc. 119, 2965–2972.
[15] Galat, A. and Goldberg, I.H. (1990) Nucleic Acids Res. 25, 2093–2099.
[16] Hensens, O.D., Giner, J.L. and Goldberg, I.H. (1989) J. Am. Chem. Soc. 111, 3295–3299.
[17] Zein, N., Colson, K.L., Leet, J.E., Schroeder, D.R., Solomon, W. and Doyle, T.W. (1993) Proc. Natl. Acad. Sci. USA 90, 2822–2826.
[18] Ando, T., Ishii, M., Kajiura, T., Kameyama, T., Miwa, K. and Sugiura, Y. (1998) Tetrahedron Lett. 39, 6495–6498.
[19] Sambrook, J. and Russell, D.W. (2001) Molecular Cloning, A Laboratory Manual, third ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[20] Kieser, T., Mervyn, J.B., Mark, B.J., Keith, C.F. and Hopwood, D.A. (2000) Practical Streptomyces Genetics. John Innes Foundation Norwich, UK.
[21] Vara, J., Lewandowska-Skarbek, M., Wang, Y.G., Donadio, S. and Hutchinson, C.R. (1989) J. Bacteriol. 171, 5872–5881.
[22] Long, P.F., Wilkinson, C.J., Bisang, C.P., Cortes, J., Dunster, N., Oliynyk, M., McCormick, E., McArthur, H., Mendez, C., Salas, J.A., Staunton, J. and Leadlay, P.F. (2002) Mol. Microbiol. 43, 1215–1225.
[23] Aparicio, J.F., Molnar, I., Schwecke, T., Konig, A., Haydock, S.F., Khaw, L.E., Staunton, J. and Leadlay, P.F. (1996) Gene 169, 9–16.
[24] Reeves, D.C., Murli, S., Ashley, G.W., Piagentini, M., Hutchinson, C.R. and McDaniel, R. (2001) Biochemistry 40, 15464–15470.
[25] Bevitt, D.J., Cortés, J., Haydock, S.F. and Leadlay, P.F. (1992) Eur. J. Biochem. 204, 39–49.
[26] Pan, H., Tsai, S.C., Meadows, E.S., Miercke, L.J.W., Keatinge-Clay, A.T., O'Connell, J., Khosla, C. and Stroud, R.M. (2002) Structure 10, 1559–1568.
[27] Ikeda, H., Nonomiya, T. and Omura, S. (2001) J. Ind. Microbiol. Biotech. 27, 170–176.
[28] Campelo, A.B. and Gil, J.A. (2002) Microbiology 148, 51–59.
[29] Barton, D. and Nakanishi, K. (1999) in: Comprehensive Natural Products Chemistry (Sankawa, U., Ed.), vol. 1, pp. 345–365, Elsevier, Amsterdam.
[30] Spencer, B.J. and Jordan, M.P. (1992) Biochem. J. 288, 839–846.
[31] Marti, T., Hu, Z., Pohl, L.N., Shah, N.A. and Khosla, C. (2000) J. Biol. Chem. 275, 33443–33448.
[32] Fujii, I., Mori, Y., Watanabe, A., Kubo, Y., Tsuji, G. and Ebizuka, Y. (2000) Biochemistry 39, 8853–8858.
[33] Beck, J., Ripka, S., Siegner, A., Schiltz, E. and Schweizer, E. (1990) Eur. J. Biochem. 192, 487–498.